

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**



Europäisches Patentamt  
European Patent Office  
Office européen des brevets



(11) Publication number: **0 492 570 A1**

(12)

## EUROPEAN PATENT APPLICATION

(21) Application number: 91122100.0

(51) Int. Cl.<sup>5</sup>: C12Q 1/68, C09B 15/00,  
C07D 221/12, C07H 21/00,  
C07C 245/20

(22) Date of filing: 23.12.91

(30) Priority: 24.12.90 US 633730

(43) Date of publication of application:  
01.07.92 Bulletin 92/27

(64) Designated Contracting States:  
DE FR GB IT

(71) Applicant: ENZO DIAGNOSTICS, INC.  
c/o Enzo Biochem, Inc., 60 Executive  
Boulevard  
Farmingdale, NY 11735(US)

(72) Inventor: Stavrianopoulos, Jannis G., Ph. D.  
519 Stafford Court, Unit G.  
Bel Mar, CA 92014(US)

(74) Representative: Vossius & Partner  
Siebertstrasse 4 P.O. Box 86 07 67  
W-8000 München 86(DE)

(54) Method for detecting a target polynucleotide in a sample using a background reducing reagent and composition and kit comprising such a reagent.

(57) The present invention provides a method for detecting the presence of a target polynucleotide in a sample. The method comprises the steps of (a) contacting the sample under hybridizing conditions with (i) a single-stranded polynucleotide probe capable of hybridizing to the target polynucleotide and comprising a polynucleotide and at least one intercalating molecule attached to a nucleotide of the polynucleotide by means of a linker arm, and (ii) a background-reducing reagent which chemically modifies the intercalating molecule when the probe to which it is attached is single-stranded; and (b) detecting a property change resulting from the intercalation of the intercalating molecule into a target-probe hybrid, thereby detecting the target polynucleotide. The intercalating molecule which is part of the polynucleotide probe induces a change in a property, in either the probe, the target polynucleotide or a target-probe hybrid. The property change can be detected, for example, by means of a generated signal which can be identified or quantified. The present invention can be employed in a heterogeneous (two step or two phase) assay using a support to immobilize the target or probe, and a washing step, and in a homogeneous (one step or one phase) assay using a hybridization solution. Also provided are a composition and a nucleic acid hybridization kit useful for detecting the presence of a target polynucleotide in a sample.

EP 0 492 570 A1

This invention provides a method for detecting the presence of a target polynucleotide in a sample by employing a background reducing reagent which reduces the signal generated by unhybridized background material. This invention also provides a composition and a nucleic acid hybridization kit using such a background reducing reagent.

Polynucleotide hybridization assays using a polynucleotide probe for verifying the presence of a target polynucleotide analyte are well known in the art. Hybridization is based on complementary base-pairing between target and probe sequences. The quantity of the polynucleotide probe, as compared to the target polynucleotide, is in excess with ratios of 6:1 of probe per target, or greater being employed conventionally. Furthermore, the amount of the probe which hybridizes to the target is typically a fraction, 0.1%. Therefore, it is very desirable to quench the fluorescence of the unhybridized probe so that minute quantities of the polynucleotide probe hybridized to the target can be accurately detected.

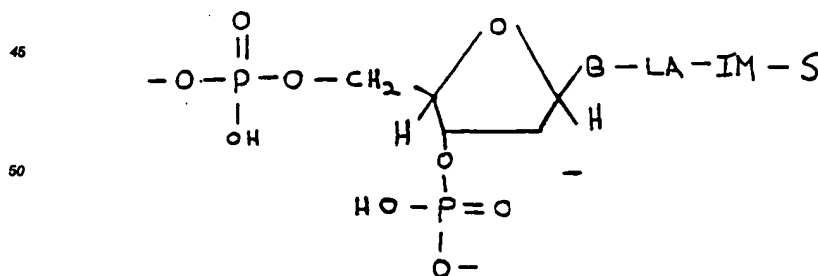
In U.S. Application Serial No. 808,757, a homogeneous assay method is disclosed in which a polynucleotide probe is used to detect the presence of a target polynucleotide in a sample. Much of the contents of Serial No. 808,757 has already been published. See, e.g., corresponding European Patent Application Publication No. 231,495, published on August 12, 1987.

The disclosed probe comprises a polynucleotide and at least a first entity and a second entity. The first entity is attached to a first nucleotide of the polynucleotide by means of a linker arm, separated by about ten nucleotides from the second entity which is attached to a second nucleotide, also by means of a linker arm. Upon hybridization of the probe to the target polynucleotide, the entities, by virtue of their characteristics, e.g., the ability to intercalate into a target-probe hybrid, provide for a property change in either the probe, the target, or both. The change in property can be detected by appropriately measuring an associated signal, e.g., radiation emission, interaction of molecular dispersion forces, such as melting temperature, and buoyant density.

It has now been discovered that the signal intensity in a hybridization assay can be significantly enhanced by contacting the hybridization reaction mixture with a background-reducing reagent which chemically modifies the intercalating groups, e.g., of an aromatic dye, when the probe to which these intercalating groups are attached remains unhybridized to target. By modifying the intercalating groups in this way, interfering signal from the unhybridized single-stranded probe can be reduced significantly, if not altogether eliminated.

The present invention provides a method for detecting the presence of a target polynucleotide in a sample. The method comprises the steps of (a) contacting the sample under hybridizing conditions with (i) a single-stranded polynucleotide probe capable of hybridizing to the target polynucleotide and comprising a polynucleotide and at least one intercalating molecule attached to a nucleotide of the polynucleotide by means of a linker arm, and (ii) a background-reducing reagent which chemically modifies the intercalating molecule when the probe to which it is attached is single-stranded; and (b) detecting a property change caused by the intercalation of the intercalating molecule into a target-probe hybrid, thereby detecting the target polynucleotide. The intercalating molecule which is part of the polynucleotide probe induces a change in a property in either the probe, the target polynucleotide or a target-probe hybrid. The property change can be detected, for example, by a signal which can be identified or quantified.

The present invention provides a composition comprising a single-stranded polynucleotide probe having at least one moiety with the structure



wherein B represents a base selected from the group consisting of pyrimidines, purines and deazapurines, provided that whenever B comprises a pyrimidine, the sugar in the base is attached to the N<sup>1</sup> position of

the pyrimidine, and whenever B is a purine or a deazapurine, the sugar is attached to the N<sup>9</sup>-position of the purine or deazapurine. As depicted in the above structure, IM represents an intercalating molecule, LA represents a linker arm comprising at least three carbon atoms and is attached covalently or non-covalently to the intercalating molecule and S represents a background-reducing reagent which modifies the intercalating molecule when the probe attached thereto is single-stranded.

A nucleic acid hybridization kit for detecting the presence of a target polynucleotide in a sample is also provided by the present invention. The kit comprises in one or more containers (1) a single-stranded polynucleotide probe capable of hybridizing to the target polynucleotide, and comprising a polynucleotide and at least one intercalating molecule attached to a nucleotide of the polynucleotide by means of a linker arm. The intercalating molecule induces a change in a property in either said probe, said target polynucleotide or a target-probe hybrid, which property change can be detected, e.g., identified or quantified through a generated signal. The kit also comprises (2) a background-reducing reagent which modifies the intercalating molecule when the probe to which the intercalating molecule is attached is single-stranded. As an optional component, the kit comprises (3) an inactivator which renders the background-reducing reagent inactive so that no further modification of the intercalating molecule can occur.

The present invention provides a method, a composition and a hybridization kit for detecting the presence of a target polynucleotide in a sample. As used herein, the term "sample" refers to those materials on which tests are performed, and includes biological, physiological, industrial, environmental and other types of solids and liquids. Of particular interest are biological tissues, such as organ or musculoskeletal specimens or biopsies, cervical and peritoneal specimens or lavages and the like, and fluids, such as serum, plasma, urine, cerebrospinal fluid, saliva, milk, broth, and other culture media and supernatants as well as fractions of any of them. Physiological fluids of interest include infusion solutions, buffers, preservative or antimicrobial solutions and the like. Industrial liquids include fermentation media and other processing liquids used, for example, in the manufacture of pharmaceuticals, dairy products and malt beverages. Other sources of sample fluids which are tested by conventional methods are also encompassed by this term and can be assayed in accordance with the invention.

The terms "target polynucleotide" or "target" refer to any nucleic-acid containing substance whose presence or absence is to be qualitatively or quantitatively determined in a sample. The method of the present invention can be applied to the detection of target oligo- or polynucleotides (or a portion thereof) which are at least partially present in single-stranded form or can be made at least partially single-stranded. The analyte, in functional terms, is usually selected from a naturally occurring or synthetic RNA or DNA for which a complementary nucleic acid exists or can be prepared.

The terms "polynucleotide probe" or "probe" refer to any nucleic acid-containing compound or composite capable of recognizing a particular nucleic acid sequence in preference to other substances. In the majority of embodiments, the probes will be DNA hybridization assay oligo- or polynucleotide probes, such as those specific for disease-causing organisms. For examples of such probes, see the product catalog for Enzo Diagnostics, Inc., an Enzo Biochem Company, 1988.

The terms "linker arm," "linkage group," "linker" and the like refer to any of the well known bonds or compounds useful in joining functional groups and which do not substantially interfere with the characteristic properties or functions of the functional groups so joined. Examples of linker arms useful in the present invention include those described for this purpose in Ward et al., U.S. Patent No. 4,711,955; Stavrianopoulos, U.S. Patent No. 4,707,352; and Stavrianopoulos, U.S. Patent No. 4,707,440, as well as pending U.S. Application Serial No. 394,284.

The terms "intercalating molecule," "intercalator," "intercalating dye," "intercalating agent" and the like refer to those species, which in the presence of double-stranded polynucleotides, can position themselves between two adjacent base-pairs in the double strands, and further interact with the base-pairs of the double strand. The hybrids can be DNA/DNA, DNA/RNA, or RNA/RNA. The characteristic of the intercalating molecule or agent is its ability to intercalate into a polynucleotide hybrid, such as a hybrid formed between a target polynucleotide and a polynucleotide probe.

Generally, the intercalating agents are aromatic dyes. In order to intercalate into the double strands, these compounds require at least five conjugated bonds. These intercalating aromatic dyes have a planar ring structure and a distinct radiation emission, e.g., fluorescence, spectra. The emission, e.g., fluorescence emission, is indicative of the electron delocalization of the intercalating agent, and is affected by the inductive effect of substituent groups attached to the dye and by quenching agents. For a further discussion of intercalation and intercalating drugs and dyes, see *Principles of Nucleic Acid Structure*, Saenger, W., Springer-Verlag, Charles Cantor editor, New York, 1983, chapter 16, pp. 350-367.

When the intercalating molecule is dissolved in an aqueous or aqueous/organic solution, it is believed that the water in the solution significantly quenches the fluorescence of the dissolved intercalating molecule

by raising the ground-energy-state of the molecule to a level higher than when the molecule is in an organic medium. If the intercalating molecule intercalates into a polynucleotide hybrid, the molecule becomes shielded from the water. Shielding occurs because the hybrid contains a relatively hydrophobic interior (the bases) and a hydrophilic exterior (the phosphates). Water thus aggregates at the exterior of the hybrid, and not at the interior. Because the fluorescence emission of the intercalating molecule is no longer quenched by the water, the ground-energy-state shifts to a lower energy level, and the result is that the fluorescence emission maximum shifts to a longer wavelength. The fluorescence intensity of the intercalating molecule, upon intercalation, is also enhanced many fold. This shift in fluorescence emission and intensity is thus a property change that is generated by the intercalating molecule only upon hybridization of the polynucleotide portion of the polynucleotide probe to the target polynucleotide.

As described in Serial No. 808,757, a single-stranded polynucleotide probe comprising a polynucleotide and at least one intercalating molecule attached to a nucleotide can be employed to detect a target polynucleotide in a homogeneous or one-step assay. Upon hybridization of the polynucleotide portion of the polynucleotide probe to the target polynucleotide to form a target-probe hybrid, the intercalating molecule intercalates into a groove of the thus formed target-probe hybrid in between stacked base-pairs. This intercalation results in a shift in the fluorescence emission and intensity of the intercalating molecule.

The term "background-reducing reagent" refers to that class of compounds which are capable of chemically modifying the above-described intercalating molecule when the polynucleotide probe to which it is attached is in single-stranded form, i.e., unhybridized to any target polynucleotide. By modifying the intercalating molecule in this way, the background-reducing reagent minimizes or substantially eliminates any interfering signal which might be generated by the unhybridized single-stranded DNA probe labeled with the intercalating (but not intercalated) molecule.

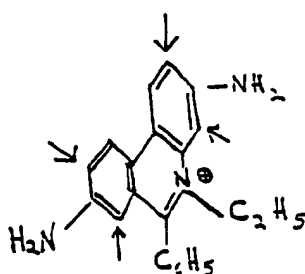
The present invention provides a method for detecting the presence of a target polynucleotide in a sample comprising the steps of: (a) contacting said sample under hybridizing conditions with (i) a single-stranded polynucleotide probe capable of hybridizing to said target polynucleotide said probe comprising a polynucleotide and at least one intercalating molecule attached to a nucleotide of said polynucleotide by means of a linker arm, wherein said intercalating molecule induces a change in a property in either said probe, said target polynucleotide or both, and (ii) a background-reducing reagent which chemically modifies said intercalating molecule when the probe attached thereto is single-stranded; and (b) detecting said property change, thereby detecting said target polynucleotide. The method can also be carried out by treating the target polynucleotide in the sample so as to render it in substantially single-stranded form prior to contacting the sample with the polynucleotide probe.

In another aspect of this method, a further step of adding an inactivator to the hybridizing reaction mixture can be performed which will render the background-reducing reagent inactive prior to detection of the property change. The inactivator works in the following manner in accordance with the present invention. The intercalating molecule is always in equilibrium between being bound to the double helix and in free form outside of the double helix. In the case where the intercalating molecule is outside of the double helix, it will be inactivated by the background-reducing reagent. To prevent a decrease in the signal e.g., fluorescence, due to reaction with the background-reducing reagent, and because the reaction of the reagent with the unbound ethidium (not intercalated) is a very fast reaction, it is useful after a certain period of time following hybridization to inactivate the background-reducing reagent. This is achieved by reacting it with a primary amine which forms a linear triazine, or using a diazo compound in the case of aromatic amines, or phenols which form diazo compounds. Merely by way of example, the inactivator can be selected from any of the group consisting of aliphatic primary amines; an aromatic phenol and an aromatic amine, or a combination of any of the foregoing. Among the aliphatic primary amines suitable as inactivators are the following: glycine, alanine, arginine, asparagine, aspartic acid, cysteine, cystine, glutamic acid, glutamine, histidine, hydroxylysine, isoleucine, leucine, lysine, methionine, serine, threonine and valine, or a combination of any of the foregoing. All of these amines are widely available from a number of commercial sources. Preferred as an inactivator in this invention is glycine and histidine. Among suitable activators selected from phenols and aromatic amines are aniline, phenol and naphthol.

The property change which results from intercalation of the double-stranded nucleic acid by the intercalating molecule takes a number of diverse forms. For example, the radiation emission of the hybrid will change as a result of intercalation. A particularly useful feature in this respect is radiation emission in the form of fluorescence, which can be conventionally measured using, for example, fluorometers which are widely available in the art. Among other changes in property which occur as a result of intercalation are interactions of molecular dispersion forces, buoyant density, melting temperature, and helical length, or a combination of any of the foregoing property changes. Especially preferred property changes are those which generate a detectable signal, e.g., fluorescence.

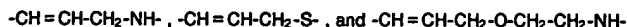
It is not always necessary in practicing the present invention to bind the intercalating molecule to the polynucleotide probe. If an assay is being performed using both single-stranded target or sample and a single-stranded polynucleotide probe, the intercalating molecule need not be bound to the probe. Instead a free form of the intercalating molecule could be used, which would intercalate into any target-probe hybrid.

Intercalating molecules useful in the practice of the present invention comprise a number of compounds. These intercalating compounds include, for example, aromatic dyes selected from phenanthridines, acridines and anthrocyclines, or combinations thereof. Useful and even preferred phenanthridines include ethidium, propidium, dimidium and phenidium, or combinations thereof. All are commercially available or can be manufactured using known methodology. Preferred is ethidium, available, for example from Sigma. St. Louis, MO. When a diazonium salt is added to ethidium, e.g., ethidium bromide, there are four possible points of attack by the diazonium salt as shown below:



Suitable members from the propidium group of compounds include, by way of illustration, 5-(4'-thiobutyl)-3,8-diamino-6-phenylphenanthridine and 5-(3'-thiopropyl)-3,8-diamino-6-phenylphenanthridine, or a combination thereof. Both are available from commercial sources, e.g., Enzo Biochem, Inc., New York, NY. Among the acridine compounds useful in the practice of the present invention is acridine orange, available commercially, for example, from Fisher Chemicals, Fairlawn, NJ.

The intercalating molecule is attached to at least one nucleotide in the polynucleotide probe by means of a linker arm, described above in U.S. Patent Nos. 4,711,955; 4,707,352; and 4,707,440, *supra*. The linker arm is typically attached to a base moiety of the polynucleotide probe, although other points of attachment can be used. The base can be selected from the group consisting of purines, pyrimidines and deazapurines, or combinations thereof. In the case of purines, these can comprise adenine or guanine. The linker arm is attached to the 8-position or exocyclic 6-amino position when the purine comprises adenine, or to the 8-position when the purine comprises guanine. Where the base comprises a deazapurine, the linker arm can be attached to the 7 or 8-position of the base. Furthermore, in the case of pyrimidines, these comprise uracil or cytosine. The linker arm is usually attached to the 5 or 6-position when the pyrimidine comprises uracil, or at the 5, 6, or exocyclic 4-amino position when the pyrimidine comprises cytosine. In other embodiments, the linker arm comprises at least 3 carbon atoms and further comprises a double bond at an alpha position relative to the base. By way of example, the linker arm can be selected from any of the following structures:



Preferred is the case where the linker arm comprises allylamine, especially where the allylamine is attached to the 5-position of uracil.

The linker arm can be attached to the base moiety in one fragment, or it can be synthesized by first attaching a first fragment to the base, and then attaching a second fragment to the first fragment. Such fragments are described in the aforementioned U.S. Application Serial No. 808,757.

Compounds which can be used as background-reducing reagents in accordance with the instant invention include a number of members which comprise the diazonium salts. Suitable diazonium salts are the following: 4-nitrobenzenediazonium salts and derivatives thereof. Preferred is 4-nitrodiazonium salt. In other preferred aspects, the 4-nitrodiazonium salt can be halogen substituted, for example, by chlorine or bromine, or a combination thereof. In the case where the diazonium salt is chlorine substituted, especially useful are the following: 3-chloro-4-nitrobenzenediazonium salt, 3,5-dichloro-benzenediazonium salt, 2,6-

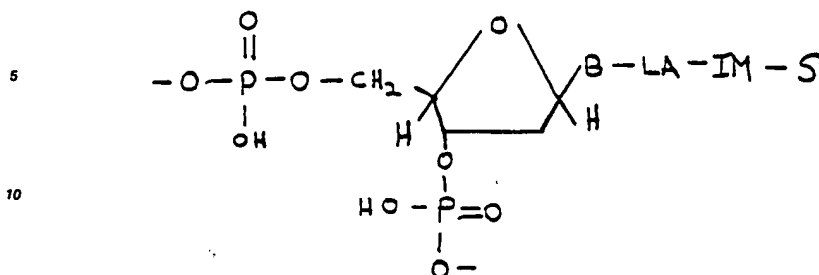
dichloro-benzenediazonium salt, 2,3,5,6-tetrachloro-benzenediazonium salt, 3,5,6-trichloro-benzenediazonium salt, 2,3,5-trichloro-benzenediazonium salt and 2,3,6-trichloro-benzenediazonium salt, or a combination of any of the foregoing. These derivatives can be prepared by reducing 4-nitroaniline (available from Aldrich Chemicals, Milwaukee, WI) to an amine and subsequent diazotization with nitrous acid in aqueous (50%) DMSO.

This invention further provides a method for detecting the presence of a target polynucleotide in a sample comprising the steps of: a) contacting the target polynucleotide with (i) a single-stranded polynucleotide probe, which probe comprises a polynucleotide and at least a first intercalating molecular entity and a second intercalating molecular entity. The first intercalating molecular entity is attached to a first nucleotide of the polynucleotide by means of a first linker arm, and the second intercalating molecular entity is attached to a second nucleotide by means of a second linker arm. The first and said second nucleotides are separated by a number of nucleotides sufficient for the provision of target specificity to the polynucleotide. The intercalating molecular entities induce upon hybridization of the polynucleotide probe to the target polynucleotide, a change in a property either in the polynucleotide probe, in the target polynucleotide, or in both. The contacting step also includes the addition of (ii) a background-reducing reagent which chemically modifies the intercalating molecular entities when the probe attached thereto is single-stranded. The method further comprises b) forming a hybrid comprising the polynucleotide probe and the target polynucleotide; and c) detecting the presence of the target polynucleotide by means of the property change. Further embodiments of the components and elements used in the aforementioned method are described earlier in this application, or are described in Serial No. 808,757.

The method described in this invention can be employed in both heterogeneous (two phase or two step) assays and homogeneous (one phase or one step) assays. In the case of the former, a solution of the polynucleotide probe bound with the intercalating molecule is added onto a solid support to which the sample or target polynucleotide has been attached. Following hybridization between the probe and target, the support is washed to further remove any unhybridized probe, and thereafter the background reducing reagent is added to "kill" fluorescence from the non-hybridized probe. This can be followed by further washing, if desired, or washing can be eliminated altogether. In any case, in the two phase reaction system, following hybridization between target and probe, the excess probe with the intercalating (but not intercalated) molecule bound thereto is inactivated (quenched) by the addition of the background-reducing reagent. After washing, signal intensity, or some other property change is determined, which indicates the presence of target. Alternatively, the polynucleotide probe with the attached intercalating molecule can be feed to a solid support, and a solution containing the target polynucleotide can be added to the support to effectuate hybridization between the target and probe. The background-reducing reagent can then be added to the support shortly after hybridization between the probe and any target contained in the sample.

It has been found that when an intercalating molecule, such as ethidium bromide, is attached to a single-stranded polynucleotide probe, there is a significant enhancement in signal generation, measured conveniently as fluorescence, over the free ethidium unbound in solution after a background-reducing reagent is added to the reaction solution. Moreover, it has been found that when the ethidium is bound and intercalated into double-stranded DNA, there is a further significant enhancement of signal, using a background-reducing reagent in accordance with this invention. Taking these two observations together, it is estimated that by employing a background-reducing reagent to chemically modify an intercalating molecule attached to a single-stranded polynucleotide probe, the decrease in interfering signal from non-specific material, e.g., unhybridized single-stranded probes, can be as high as 10,000 over the conventional assay.

The present invention provides a composition comprising a single-stranded polynucleotide probe having at least one moiety with the structure



wherein B represents a base selected from the group consisting of pyrimidines, purines and deazapurines, provided that whenever B comprises a pyrimidine, the sugar in the base is attached to the N<sup>1</sup>-position of the pyrimidine, and whenever B is a purine or a deazapurine, the sugar is attached to the N<sup>9</sup>-position of the purine or deazapurine. Furthermore, in the above composition, IM represents an intercalating molecule. LA represents a linker arm comprising at least three carbon atoms and is attached covalently or non-covalently to the intercalating molecule, and S represents a background reducing reagent which modifies the intercalating molecule when the probe attached thereto is single-stranded. All of these moieties in the above composition have been described hereinabove.

The present invention further provides a nucleic acid hybridization kit for detecting the presence of a target polynucleotide in a sample. The kit comprises in one or more containers or in packaged combination the following: (1) a single-stranded polynucleotide probe which is hybridizable to the target polynucleotide. The probe comprises a polynucleotide and at least one intercalating molecule attached to a nucleotide of the polynucleotide by means of a linker arm. The intercalating molecule induces a change in a property in either the probe, the target polynucleotide or both; and (2) a background-reducing reagent which modifies the intercalating molecule when the probe attached thereto is single-stranded. Optionally included in this kit is (3) an inactivator which renders the background-reducing reagent inactive. The components and elements of this kit have been described hereinabove.

The examples which follow are set forth to illustrate various aspects of the present invention but are not intended to limit in any way its scope as more particularly set forth in the claims.

#### EXAMPLE 1: Comparison of emission between single-stranded DNA bound with ethidium bromide and an intercalated DNA/RNA hybrid.

In this example, a comparison was made in terms of fluorescence emission between single-stranded DNA containing intercalated molecules and a double-stranded DNA/RNA hybrid. A standard DNA solution was formulated (10  $\mu$ g/260 nm) which contained 30 mer thymidine in which every fourth nucleotide was labeled with ethidium bromide. 20  $\mu$ l from the solution was removed and combined with 0.1 molar sodium acetate buffer at pH 7.0. 5  $\mu$ l of 0.1 molar diazonium salt prepared by conventional methodology. The diazonium salt was prepared from 4-nitroaniline (Aldrich Chemicals, Milwaukee, WI) which was reduced to the corresponding amine, and subsequently diazotized with nitrous acid in 50% (aqueous) DMSO. The reaction was allowed to proceed for five minutes after which time fluorescence was measured under an ultraviolet lamp using a UV lamp from UVP, Inc., San Gabriel, CA 91778. Alternatively, fluorescence could be measured with a fluorimeter, e.g., Perkin-Elmer, South Plainfield, NJ, if equipped with the proper UV lamp. Fluorescence in the reaction mixture was observed to disappear during the five minute waiting period.

20  $\mu$ l from the same standard DNA solution was taken, placed in 0.1 molar sodium acetate buffer (pH 7.0) and combined with an equivalent molar amount of polyribonucleic (polyA) acid. 5  $\mu$ l of the 0.1 molar diazonium salt used in the first part of this example was then added to the reaction mixture. Reaction was allowed to proceed for five minutes after which time fluorescence was observed to persist in the reaction mixture.

The results of this example demonstrate that intercalation of ethidium bromide into a double-stranded nucleic acid prevents chemical modification by the diazonium salt. The protection of the intercalating molecule by the double-stranded nucleic acid is evidenced by the persistence of fluorescence in the reaction mixture containing the diazonium salt and the double-stranded DNA/RNA hybrid where the



ethidium bromide of the DNA strand intercalates into the double helix. Another class of compounds which intercalates into double-stranded helices are the acridines. Acridine orange was used in this next example to investigate the behavior of this particular aromatic dye against the reaction with a background-reducing reagent.

5

**EXAMPLE 2: Comparison between double-stranded DNA intercalated with acridine orange and unbound acridine orange (free in solution).**

In this example, acridine orange was employed as an intercalating molecule to illustrate the differences between the unbound intercalating molecule (free in solution) and double-stranded DNA which is intercalated with the molecule.

0.1 mg per ml of calf thymus double-stranded DNA was combined with traces of acridine orange (in sodium acetate buffer, pH 7.0). The acridine orange was obtained from Fisher Chemicals, Fairlawn, New Jersey. After the addition of the acridine, an intensive yellow fluorescence appeared in the reaction mixture.

As a control, a tube was set up without the aforementioned DNA using traces of acridine orange in sodium acetate buffer, pH 7.0. To the buffered solution was added 5  $\mu$ l of 0.1 molar of the diazonium salt, p-nitrobenzenediazonium chloride, which was prepared by reducing 4-nitroaniline (Aldrich Chemicals, Milwaukee, WI) to an amine and subsequent diazotization with nitrous acid in aqueous 50% DMSO.

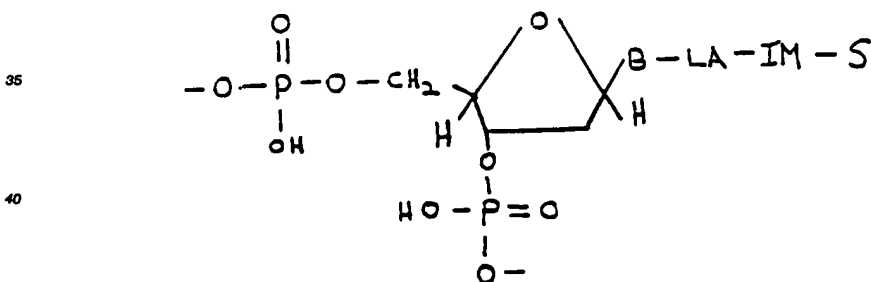
Reaction was allowed to proceed for five minutes and fluorescence was observed in the reaction solution. After the addition of the diazonium salt, the fluorescence control disappeared, in contrast to the DNA containing solution where the fluorescence persisted. These observations indicate that as an intercalating molecule, acridine orange is protected by the double-stranded DNA, and hints to the wide application of diazonium salts with fluorescent intercalators.

**Claims**

1. A method for detecting the presence of a target polynucleotide in a sample comprising the steps of:
  - (a) contacting said sample under hybridizing conditions with (i) a single-stranded polynucleotide probe capable of hybridizing to said target polynucleotide, said probe comprising a polynucleotide and at least one intercalating molecule attached to a nucleotide of said polynucleotide by means of a linker arm, wherein said intercalating molecule induces a change in a property in either said probe, said target polynucleotide or both, and (ii) a background-reducing reagent which chemically modifies said intercalating molecule when the probe attached thereto is single-stranded; and
  - (b) detecting said property change, thereby detecting said target polynucleotide.
2. The method of claim 1 wherein said target polynucleotide is in double-stranded form and said method further comprises the step of rendering said target polynucleotide in substantially single-stranded form prior to said contacting step.
3. The method of claim 1 or 2 further comprising the step of adding an inactivator which renders said background-reducing reagent inactive prior to detecting the signal.
4. The method of claim 3 wherein said inactivator is selected from the group consisting of an aliphatic primary amine, a phenol and an aromatic amine, or a combination of any of the foregoing.
5. The method according to any one of claims 1 to 4 wherein said property change is selected from the group consisting of radiation emission, interaction of molecular dispersion forces, buoyant density, melting temperature and helical length, or a combination of any of the foregoing.
6. The method according to any one of claims 1 to 4 wherein said property change comprises a signal.
7. The method according to any one of claims 1 to 6 wherein said intercalating molecule comprises an aromatic dye.
8. The method according to any one of claims 1 to 7 wherein said linker arm is attached to a base moiety of said polynucleotide probe.
9. The method of claim 8 wherein said base is selected from the group consisting of purines, pyrimidines

and deazapurine, or a combination of any of the foregoing.

10. The method of claim 8 or 9 wherein said linker arm comprises at least 3 carbon atoms.
- 5 11. The method according to any one of claims 8 to 10 wherein said linker arm is attached to the base moiety in one fragment.
12. The method according to any one of claims 8 to 11 wherein said linker arm is synthesized by first attaching a first fragment to the base moiety and then attaching a second fragment to the first fragment.
- 10 13. The method according to any one of claims 1 to 12 wherein said background-reducing reagent comprises a diazonium salt.
14. A method for detecting the presence of a target polynucleotide in a sample comprising the steps of:
  - 15 a) contacting said target polynucleotide with (i) a single-stranded polynucleotide probe, said polynucleotide probe comprising a polynucleotide and at least a first intercalating molecular entity and a second intercalating molecular entity, wherein said first intercalating molecular entity is attached to a first nucleotide of said polynucleotide by means of a first linker arm, and said second intercalating molecular entity is attached to a second nucleotide by means of a second linker arm, said first and said second nucleotides being separated by a number of nucleotides sufficient for the provision of target specificity to said polynucleotide, said intercalating molecular entities inducing upon hybridization of said polynucleotide probe to said target polynucleotide, a change in a property either in the polynucleotide probe, in the target polynucleotide, or in both; and (ii) a background-reducing reagent which chemically modifies said intercalating molecular entities when the probe attached thereto is single-stranded;
  - 20 b) forming a hybrid comprising said polynucleotide probe and said target polynucleotide; and
  - 25 c) detecting the presence of said target polynucleotide by means of said property change.
15. A composition comprising a single-stranded polynucleotide probe having at least one moiety with the structure



- 45 wherein B represents a base selected from the group consisting of pyrimidines, purines and deazapurines, provided that whenever B comprises a pyrimidine, the sugar in said base is attached to the N<sup>1</sup>-position of the pyrimidine, and whenever B is a purine or a deazapurine, the sugar is attached to the N<sup>9</sup>-position of the purine or deazapurine, wherein IM represents an intercalating molecule, LA represents a linker arm comprising at least three carbon atoms and is attached covalently or non-covalently to said intercalating molecule, and S represents a background-reducing reagent which modifies said intercalating molecule when the probe attached thereto is single-stranded.
16. The composition of claim 15 wherein said intercalating molecule comprises an aromatic dye.
- 55 17. The composition of claim 15 or 16 wherein said background-reducing reagent comprises a diazonium salt.

18. A nucleic acid hybridization kit for detecting the presence of a target polynucleotide in a sample, said kit comprising in one or more containers:

(1) a single-stranded polynucleotide probe which is hybridizable to said target polynucleotide, said probe comprising a polynucleotide and at least one intercalating molecule attached to a nucleotide of said polynucleotide by means of a linker arm, wherein said intercalating molecule induces a

change in a property in either said probe, said target polynucleotide or both;

(2) a background-reducing reagent which modifies said intercalating molecule when the probe attached thereto is single-stranded;

optionally with

(3) an inactivator which renders the background-reducing reagent inactive.



European Patent  
Office

# EUROPEAN SEARCH REPORT

Application Number

EP 91 12 2100

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
D, Y	EP-A-0 231 495 (ENZO BIOCHEM., INC.) * the whole document *	1-18	C12Q1/68 C09B15/00 C07D221/12 C07H21/00 C07C245/20
Y	WO-A-8 902 476 (NL TECHNOLOGY VENTURES) * the whole document * especially * claim 5 *	1-18	
Y	FR-A-1 295 153 (MAY AND BAKER LIMITED) 24 Apr 11 1962 * example *	13, 17	
Y	DE-B-1 203 781 (MAY AND BAKER LIMITED) 28 October 1965 * examples *	13, 17	
Y	CHEMICAL ABSTRACTS, vol. 107, 1987, Columbus, Ohio, US; abstract no. 96576U, BHAVSAR, M. D. ET AL.: 'Synthesis of acridine derivatives. I. Meerwein arylation of acridine' page 677; column 2; * abstract * & MAN MADE TEXT, INDIA vol. 29, no. 10, 1986, pages 485 - 487;	13, 17	TECHNICAL FIELDS SEARCHED (Int. Cl.5)  C12Q
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 24 MARCH 1992	Examiner MOLINA GALAN E.
<b>CATEGORY OF CITED DOCUMENTS</b> X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons @ : member of the same patent family, corresponding document			

EPF FORM 1300 (12.92) (P.0001)



Europäisches Patentamt  
European Patent Office  
Office européen des brevets

(11) **EP 0 492 570 B1**

(12) **EUROPEAN PATENT SPECIFICATION**

(43) Date of publication:  
01.07.1992 Bulletin 1992/27

(51) Int. Cl.<sup>7</sup>: **C12Q 1/68, C09B 15/00,  
C07D 221/12, C07H 21/00,  
C07C 245/20**

(21) Application number: **91122100.0**

(22) Date of filing: **23.12.1991**

(84) Designated Contracting States:  
**DE FR GB IT**

**Bel Mar, CA 92014 (US)**

(30) Priority: **24.12.1990 US 633730**

(74) Representative:  
**VOSSIUS & PARTNER  
Postfach 86 07 67  
81634 München (DE)**

(72) Inventor: **Stavrianopoulos, Jannis G., Ph. D.**

(54) **Method for detecting a target polynucleotide in a sample using a background reducing reagent and composition and kit comprising such a reagent**

**EP 0 492 570 B1**

## Description

[0001] This invention provides a method for detecting the presence of a target polynucleotide in a sample by employing a background reducing reagent which reduces the signal generated by unhybridized background material. This invention also provides a composition and a nucleic acid hybridization kit using such a background reducing reagent.

[0002] Polynucleotide hybridization assays using a polynucleotide probe for verifying the presence of a target polynucleotide analyte are well known in the art. Hybridization is based on complementary base-pairing between target and probe sequences. The quantity of the polynucleotide probe, as compared to the target polynucleotide, is in excess with ratios of 6:1 of probe per target, or greater being employed conventionally. Furthermore, the amount of the probe which hybridizes to the target is typically a fraction, 0.1%. Therefore, it is very desirable to quench the fluorescence of the unhybridized probe so that minute quantities of the polynucleotide probe hybridized to the target can be accurately detected.

[0003] In EP-A-231 495 a homogeneous assay method is disclosed in which a polynucleotide probe is used to detect the presence of a target polynucleotide in a sample.

[0004] The disclosed probe comprises a polynucleotide and at least a first entity and a second entity. The first entity is attached to a first nucleotide of the polynucleotide by means of a linker arm, separated by about ten nucleotides from the second entity which is attached to a second nucleotide, also by means of a linker arm. Upon hybridization of the probe to the target polynucleotide, the entities, by virtue of their characteristics, e.g., the ability to intercalate into a target-probe hybrid, provide for a property change in either the probe, the target or both. The change in property can be detected by appropriately measuring an associated signal, e.g., radiation emission, interaction of molecular dispersion forces, such as melting temperature, and buoyant density.

[0005] It has now been discovered that the signal intensity in a hybridization assay can be significantly enhanced by contacting the hybridization reaction mixture with a background-reducing reagent which chemically modifies the intercalating groups, e.g., of an aromatic dye, when the probe to which these intercalating groups are attached remains unhybridized to target. By modifying the intercalating groups in this way, interfering signal from the unhybridized single-stranded probe can be reduced significantly, if not altogether eliminated.

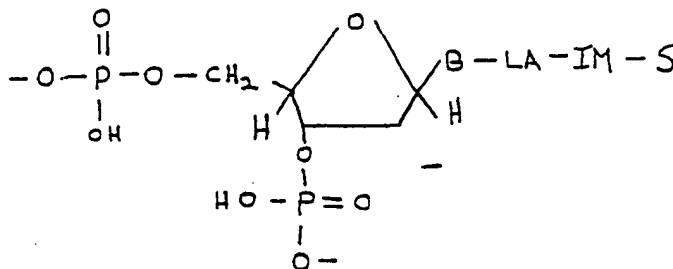
[0006] WO 89/02476 discloses homogenous diagnostic assay methods and labels with reduced background noise and increased detection sensitivity due to the difference in the degradation rates between labels on free probes and labels on hybridized probes.

[0007] The present invention provides a method for detecting the presence of a target polynucleotide in a sample. The method comprises the steps of

(a) contacting said sample under hybridizing conditions with (i) a single-stranded polynucleotide probe capable of hybridizing to said target polynucleotide, said probe comprising a polynucleotide and at least one intercalating molecule attached to a nucleotide of said polynucleotide by means of a linker arm, wherein upon hybridization of said probe to said target said intercalating molecule induces a detectable change in a property in either said probe, said target polynucleotide or both, and (ii) a background-reducing reagent which chemically modifies said intercalating molecule when the probe attached thereto is single-stranded in such a way that any interfering signal from the unhybridized single stranded DNA probe is minimized or substantially eliminated; and

(b) detecting said property change, thereby detecting said target polynucleotide.

[0008] The present invention provides a composition comprising a single-stranded polynucleotide probe having at least one moiety with the structure



wherein B represents a base selected from the group consisting of pyrimidines, purines and deazapurines, provided that whenever B comprises a pyrimidine, the sugar in said base is attached to the N<sup>1</sup>'a-position of the pyrimidine, and

EP 0 492 570 B1

whenever B is a purine or a deazapurine, the sugar is attached to the N<sup>9</sup>-position of the purine or deazapurine, wherein IM represents an intercalating molecule which upon hybridization of the probe to its target induces a detectable change in a property in either said probe, said target or both, LA represents a linker arm comprising at least three carbon atoms and is attached covalently or non-covalently to said intercalating molecule, and S represents a background-reducing reagent which modifies said intercalating molecule when the probe attached thereto is single-stranded in such a way that any interfering signal from the unhybridized single stranded DNA probe is minimized or substantially eliminated.

[0009] A nucleic acid hybridization kit for detecting the presence of a target polynucleotide in a sample is also provided by the present invention. The kit comprises in one or more containers

(1) a single-stranded polynucleotide probe which is hybridizable to said target polynucleotide said probe comprising a polynucleotide and at least one intercalating molecule attached to a nucleotide of said polynucleotide by means of a linker arm, wherein said intercalating molecule induces a change in a property in either said probes said target polynucleotide or both;

(2) a background-reducing reagent which modifies said intercalating molecule when the probe attached thereto is single-stranded in such a way that any interfering signal from the unhybridized single stranded DNA probe is minimized or substantially eliminated; optionally with

(3) an inactivator which renders the background-reducing reagent inactive.

[0010] The present invention provides a method, a composition and a hybridization kit for detecting the presence of a target polynucleotide in a sample. As used herein, the term "sample" refers to those materials on which tests are performed, and includes biological, physiological, industrial, environmental and other types of solids and liquids. Of particular interest are biological tissues, such as organ or musculoskeletal specimens or biopsies, cervical and peritoneal specimens or lavages and the like, and fluids, such as serum, plasma, urine, cerebrospinal fluid, saliva, milk, broth, and other culture media and supernatants as well as fractions of any of them. Physiological fluids of interest include infusion solutions, buffers, preservative or antimicrobial solutions and the like. Industrial liquids include fermentation media and other processing liquids used, for example, in the manufacture of pharmaceuticals, dairy products and malt beverages. Other sources of sample fluids which are tested by conventional methods are also encompassed by this term and can be assayed in accordance with the invention.

[0011] The terms "target polynucleotide" or "target" refer to any nucleic-acid containing substance whose presence or absence is to be qualitatively or quantitatively determined in a sample. The method of the present invention can be applied to the detection of target oligo- or polynucleotides (or a portion thereof) which are at least partially present in single-stranded form or can be made at least partially single-stranded. The analyte, in functional terms, is usually selected from a naturally occurring or synthetic RNA or DNA for which a complementary nucleic acid exists or can be prepared.

[0012] The terms "polynucleotide probe" or "probe" refer to any nucleic acid-containing compound or composite capable of recognizing a particular nucleic acid sequence in preference to other substances. In the majority of embodiments, the probes will be DNA hybridization assay oligo- or polynucleotide probes, such as those specific for disease-causing organisms. For examples of such probes, see the product catalog for Enzo Diagnostics, Inc., an Enzo Biochem Company, 1988.

[0013] The terms "linker arm," "linkage group," "linker" and the like refer to any of the well known bonds or compounds useful in joining functional groups and which do not substantially interfere with the characteristic properties or functions of the functional groups so joined. Examples of linker arms useful in the present invention include those described for this purpose in Ward et al., U.S. Patent No. 4,711,955; Stavrianopoulos, U.S. Patent No. 4,707,352; and Stavrianopoulos, U.S. Patent No. 4,707,440.

[0014] The terms "intercalating molecule," "intercalator," "intercalating dye," "intercalating agent" and the like refer to those species, which in the presence of double-stranded polynucleotides, can position themselves between two adjacent base-pairs in the double strands, and further interact with the base-pairs of the double strand. The hybrids can be DNA/DNA, DNA/RNA, or RNA/RNA. The characteristic of the intercalating molecule or agent is its ability to intercalate into a polynucleotide hybrid, such as a hybrid formed between a target polynucleotide and a polynucleotide probe.

[0015] Generally, the intercalating agents are aromatic dyes. In order to intercalate into the double strands, these compounds require at least five conjugated bonds. These intercalating aromatic dyes have a planar ring structure and a distinct radiation emission, e.g., fluorescence, spectra. The emission, e.g., fluorescence emission, is indicative of the electron delocalization of the intercalating agent, and is affected by the inductive effect of substituent groups attached to the dye and by quenching agents. For a further discussion of intercalation and intercalating drugs and dyes, see Principles of Nucleic Acid Structure, Saenger, W., Springer-Verlag, Charles Cantor editor, New York, 1983, chapter 16, pp. 350-387.

[0016] When the intercalating molecule is dissolved in an aqueous or aqueous/organic solution, it is believed that the water in the solution significantly quenches the fluorescence of the dissolved intercalating molecule by raising the ground-energy-state of the molecule to a level higher than when the molecule is in an organic medium. If the intercalating molecule intercalates into a polynucleotide hybrid, the molecule becomes shielded from the water.

EP 0 492 570 B1

Shielding occurs because the hybrid contains a relatively hydrophobic interior (the bases) and a hydrophilic exterior (the phosphates). Water thus aggregates at the exterior of the hybrid, and not at the interior. Because the fluorescence emission of the intercalating molecule is no longer quenched by the water, the ground-energy-state shifts to a lower energy level, and the result is that the fluorescence emission maximum shifts to a longer wavelength. The fluorescence intensity of the intercalating molecule, upon intercalation, is also enhanced many fold. This shift in fluorescence emission and intensity is thus a property change that is generated by the intercalating molecule only upon hybridization of the polynucleotide portion of the polynucleotide probe to the target polynucleotide.

[0017] As described in EP-A-231 495, a single-stranded polynucleotide probe comprising a polynucleotide and at least one intercalating molecule attached to a nucleotide can be employed to detect a target polynucleotide in a homogeneous or one-step assay. Upon hybridization of the polynucleotide portion of the polynucleotide probe to the target polynucleotide to form a target-probe hybrid, the intercalating molecule intercalates into a groove of the thus formed target-probe hybrid in between stacked base-pairs. This intercalation results in a shift in the fluorescence emission and intensity of the intercalating molecule.

[0018] The term "background-reducing reagent" refers to that class of compounds which are capable of chemically modifying the above-described intercalating molecule when the polynucleotide probe to which it is attached is in single-stranded form, i.e., unhybridized to any target polynucleotide. By modifying the intercalating molecule in this way, the background-reducing reagent minimizes or substantially eliminates any interfering signal which might be generated by the unhybridized single-stranded DNA probe labeled with the intercalating (but not intercalated) molecule.

[0019] The present invention provides a method for detecting the presence of a target polynucleotide in a sample comprising the steps of: (a) contacting said sample under hybridizing conditions with (i) a single-stranded polynucleotide probe capable of hybridizing to said target polynucleotide, said probe comprising a polynucleotide and at least one intercalating molecule attached to a nucleotide of said polynucleotide by means of a linker arm, wherein said intercalating molecule induces a change in a property in either said probe, said target polynucleotide or both, and (ii) a background-reducing reagent which chemically modifies said intercalating molecule when the probe attached thereto is single-stranded; and (b) detecting said property change, thereby detecting said target polynucleotide. The method can also be carried out by treating the target polynucleotide in the sample so as to render it in substantially single-stranded form prior to contacting the sample with the polynucleotide probe.

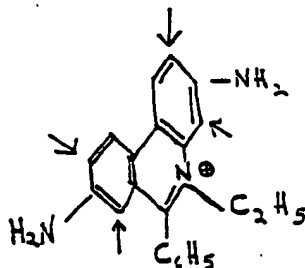
[0020] In another aspect of this method, a further step of adding an inactivator to the hybridizing reaction mixture can be performed which will render the background-reducing reagent inactive prior to detection of the property change. The inactivator works in the following manner in accordance with the present invention. The intercalating molecule is always in equilibrium between being bound to the double helix and in free form outside of the double helix. In the case where the intercalating molecule is outside of the double helix, it will be inactivated by the background-reducing reagent. To prevent a decrease in the signal e.g., fluorescence, due to reaction with the background-reducing reagent, and because the reaction of the reagent with the unbound ethidium (not intercalated) is a very fast reaction, it is useful after a certain period of time following hybridization to inactivate the background-reducing reagent. This is achieved by reacting it with a primary amine which forms a linear triazine, or using a diazo compound in the case of aromatic amines, or phenols which form diazo compounds. Merely by way of example, the inactivator can be selected from any of the group consisting of aliphatic primary amines, an aromatic phenol and an aromatic amine, or a combination of any of the foregoing. Among the aliphatic primary amines suitable as inactivators are the following: glycine, alanine, arginine, asparagine, aspartic acid, cysteine, cystine, glutamic acid, glutamine, histidine, hydroxylysine, isoleucine, leucine, lysine, methionine, serine, threonine and valine, or a combination of any of the foregoing. All of these amines are widely available from a number of commercial sources. Preferred as an inactivator in this invention is glycine and histidine. Among suitable activators selected from phenols and aromatic amines are aniline, phenol and naphthol.

[0021] The property change which results from intercalation of the double-stranded nucleic acid by the intercalating molecule takes a number of diverse forms. For example, the radiation emission of the hybrid will change as a result of intercalation. A particularly useful feature in this respect is radiation emission in the form of fluorescence, which can be conventionally measured using, for example, fluorometers which are widely available in the art. Among other changes in property which occur as a result of intercalation are interactions of molecular dispersion forces, buoyant density, melting temperature, and helical length, or a combination of any of the foregoing property changes. Especially preferred property changes are those which generate a detectable signal, e.g., fluorescence.

[0022] It is not always necessary in practicing the present invention to bind the intercalating molecule to the polynucleotide probe. If an assay is being performed using both single-stranded target or sample and a single-stranded polynucleotide probe, the intercalating molecule need not be bound to the probe. Instead a free form of the intercalating molecule could be used, which would intercalate into any target-probe hybrid.

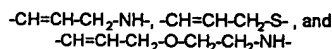
[0023] Intercalating molecules useful in the practice of the present invention comprise a number of compounds. These intercalating compounds include, for example, aromatic dyes selected from phenanthridines, acridines and anthrocyclines, or combinations thereof. Useful and even preferred phenanthridines include ethidium, propidium, dimidium and phenidium, or combinations thereof. All are commercially available or can be manufactured using known methodology. Preferred is ethidium, available, for example from Sigma. St. Louis, MO. When a diazonium salt is added to ethidium, e.g., ethidium bromide, there are four possible points of attack by the diazonium salt as shown below:





[0024] Suitable members from the propidium group of compounds include, by way of illustration, 5-(4'-thiobutyl)-3,8-diamino-6-phenylphenanthridine and 5-(3'-thiopropyl)-3,8-diamino-6-phenylphenanthridine, or a combination thereof. Both are available from commercial sources, e.g., Enzo Biochem, Inc., New York, NY. Among the acridine compounds useful in the practice of the present invention is acridine orange, available commercially, for example, from Fisher Chemicals, Fairlawn, NJ.

[0025] The intercalating molecule is attached to at least one nucleotide in the polynucleotide probe by means of a linker arm, described above in U.S. Patent Nos. 4,711,955; 4,707,352; and 4,707,440, *supra*. The linker arm is typically attached to a base moiety of the polynucleotide probe, although other points of attachment can be used. The base can be selected from the group consisting of purines, pyrimidines and deazapurines, or combinations thereof. In the case of purines, these can comprise adenine or guanine. The linker arm is attached to the 8-position or exocyclic 6-amino position when the purine comprises adenine, or to the 8-position when the purine comprises guanine. Where the base comprises a deazapurine, the linker arm can be attached to the 7 or 8-position of the base. Furthermore, in the case of pyrimidines, these comprise uracil or cytosine. The linker arm is usually attached to the 5 or 6-position when the pyrimidine comprises uracil, or at the 5, 6, or exocyclic 4-amino position when the pyrimidine comprises cytosine. In other embodiments, the linker arm comprises at least 3 carbon atoms and further comprises a double bond at an alpha position relative to the base. By way of example, the linker arm can be selected from any of the following structures:



[0026] Preferred is the case where the linker arm comprises allylamine, especially where the allylamine is attached to the 5-position of uracil.

[0027] The linker arm can be attached to the base moiety in one fragment, or it can be synthesized by first attaching a first fragment to the base, and then attaching a second fragment to the first fragment. Such fragments are described in the aforementioned EP-A-231 495.

[0028] Compounds which can be used as background-reducing reagents in accordance with the instant invention include a number of members which comprise the diazonium salts. Suitable diazonium salts are the following: 4-nitrobenzenediazonium salts and derivatives thereof. Preferred is 4-nitrodiazonium salt. In other preferred aspects, the 4-nitrodiazonium salt can be halogen substituted, for example, by chlorine or bromine, or a combination thereof. In the case where the diazonium salt is chlorine substituted, especially useful are the following: 3-chloro-4-nitrobenzenediazonium salt, 3,5-dichlorobenzenediazonium salt, 2,6-dichloro-benzenediazonium salt, 2,3,5,6-tetrachloro-benzenediazonium salt, 3,5,6-trichlorobenzenediazonium salt, 2,3,5-trichloro-benzenediazonium salt and 2,3,6-trichloro-benzenediazonium salt, or a combination of any of the foregoing. These derivatives can be prepared by reducing 4-nitroaniline (available from Aldrich Chemicals, Milwaukee, WI) to an amine and subsequent diazotization with nitrous acid in aqueous (50%) DMSO.

[0029] This invention further provides a method for detecting the presence of a target polynucleotide in a sample comprising the steps of: a) contacting the target polynucleotide with (i) a single-stranded polynucleotide probe, which probe comprises a polynucleotide and at least a first intercalating molecular entity and a second intercalating molecular entity. The first intercalating molecular entity is attached to a first nucleotide of the polynucleotide by means of a first linker arm, and the second intercalating molecular entity is attached to a second nucleotide by means of a second linker arm. The first and said second nucleotides are separated by a number of nucleotides sufficient for the provision of target specificity to the polynucleotide. The intercalating molecular entities induce upon hybridization of the polynucleotide probe to the target polynucleotide, a change in a property either in the polynucleotide probe, in the target polynucleotide, or in both. The contacting step also includes the addition of (ii) a background-reducing reagent which chemically modifies the intercalating molecular entities when the probe attached thereto is single-stranded. The method further comprises b) forming a hybrid comprising the polynucleotide probe and the target polynucleotide; and c) detecting the presence of the target polynucleotide by means of the property change. Further embodiments of the components and elements used in the aforementioned

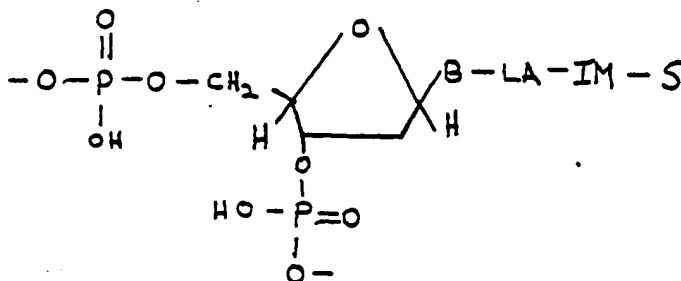
EP 0 492 570 B1

method are described earlier in this application, or are described in EP-A-231 495.

[0030] The method described in this invention can be employed in both heterogeneous (two phase or two step) assays and homogeneous (one phase or one step) assays. In the case of the former, a solution of the polynucleotide probe bound with the intercalating molecule is added onto a solid support to which the sample or target polynucleotide has been attached. Following hybridization between the probe and target, the support is washed to further remove any unhybridized probe, and thereafter, the background reducing reagent is added to "kill" fluorescence from the non-hybridized probe. This can be followed by further washing, if desired, or washing can be eliminated altogether. In any case, in the two phase reaction system, following hybridization between target and probe, the excess probe with the intercalating (but not intercalated) molecule bound thereto is inactivated (quenched) by the addition of the background-reducing reagent. After washing, signal intensity, or some other property change is determined, which indicates the presence of target. Alternatively, the polynucleotide probe with the attached intercalating molecule can be fixed to a solid support, and a solution containing the target polynucleotide can be added to the support to effectuate hybridization between the target and probe. The background-reducing reagent can then be added to the support shortly after hybridization between the probe and any target contained in the sample.

[0031] It has been found that when an intercalating molecule, such as ethidium bromide, is attached to a single-stranded polynucleotide probe, there is a significant enhancement in signal generation, measured conveniently as fluorescence, over the free ethidium unbound in solution after a background-reducing reagent is added to the reaction solution. Moreover, it has been found that when the ethidium is bound and intercalated into double-stranded DNA, there is a further significant enhancement of signal, using a background-reducing reagent in accordance with this invention. Taking these two observations together, it is estimated that by employing a background-reducing reagent to chemically modify an intercalating molecule attached to a single-stranded polynucleotide probe, the decrease in interfering signal from non-specific material, e.g., unhybridized single-stranded probes, can be as high as 10,000 over the conventional assay.

[0032] The present invention provides a composition comprising a single-stranded polynucleotide probe having at least one moiety with the structure



wherein B represents a base, selected from the group consisting of pyrimidines, purines and deazapurines, provided that whenever B comprises a pyrimidine, the sugar in the base is attached to the N1'-position of the pyrimidine, and whenever B is a purine or a deazapurine, the sugar is attached to the N9'-position of the purine or deazapurine. Furthermore, in the above composition, IM represents an intercalating molecule. LA represents a linker arm comprising at least three carbon atoms and is attached covalently or non-covalently to the intercalating molecule, and S represents a background reducing reagent which modifies the intercalating molecule when the probe attached thereto is single-stranded. All of these moieties in the above composition have been described hereinabove.

[0033] The present invention further provides a nucleic acid hybridization kit for detecting the presence of a target polynucleotide in a sample. The kit comprises in one or more containers or in packaged combination the following: (1) a single-stranded polynucleotide probe which is hybridizable to the target polynucleotide. The probe comprises a polynucleotide and at least one intercalating molecule attached to a nucleotide of the polynucleotide by means of a linker arm. The intercalating molecule induces a change in a property in either the probe, the target polynucleotide or both; and (2) a background-reducing reagent which modifies the intercalating molecule when the probe attached thereto is single-stranded. Optionally included in this kit is (3) an inactivator which renders the background-reducing reagent inactive. The components and elements of this kit have been described hereinabove.

[0034] The examples which follow are set forth to illustrate various aspects of the present invention but are not intended to limit in any way its scope as more particularly set forth in the claims.

**EXAMPLE 1: Comparison of emission between single-stranded DNA bound with ethidium bromide and an intercalated DNA/RNA hybrid.**

[0035] In this example, a comparison was made in terms of fluorescence emission between single-stranded DNA

EP 0 492 570 B1

containing intercalated molecules and a double-stranded DNA/RNA hybrid. A standard DNA solution was formulated (10 OD/260 nm) which contained 30 mer thymidine in which every fourth nucleotide was labeled with ethidium bromide. 20 ul from the solution was removed and combined with 0.1 molar sodium acetate buffer at pH 7.0. 5 ul of 0.1 molar diazonium salt prepared by conventional methodology. The diazonium salt was prepared from 4-nitroaniline (Aldrich Chemicals, Milwaukee, WI) which was reduced to the corresponding amine, and subsequently diazotized with nitrous acid in 50% (aqueous) DMSO. The reaction was allowed to proceed for five minutes after which time fluorescence was measured under an ultraviolet lamp using a UV lamp from UVP, Inc., San Gabriel, CA 91778. Alternatively, fluorescence could be measured with a fluorimeter, e.g., Perkin-Elmer, South Plainfield, NJ, if equipped with the proper UV lamp. Fluorescence in the reaction mixture was observed to disappear during the five minute waiting period.

[0038] 20 ul from the same standard DNA solution was taken, placed in 0.1 molar sodium acetate buffer (pH 7.0) and combined with an equivalent molar amount of polyribonucleic (polyA) acid. 5 ul of the 0.1 molar diazonium salt used in the first part of this example was then added to the reaction mixture. Reaction was allowed to proceed for five minutes after which time fluorescence was observed to persist in the reaction mixture.

[0037] The results of this example demonstrate that intercalation of ethidium bromide into a double-stranded nucleic acid prevents chemical modification by the diazonium salt. The protection of the intercalating molecule by the double-stranded nucleic acid is evidenced by the persistence of fluorescence in the reaction mixture containing the diazonium salt and the double-stranded DNA/RNA hybrid where the ethidium bromide of the DNA strand intercalates into the double helix. Another class of compounds which intercalates into double-stranded helices are the acridines. Acridine orange was used in this next example to investigate the behavior of this particular aromatic dye against the reaction with a background-reducing reagent.

**EXAMPLE 2: Comparison between double-stranded DNA intercalated with acridine orange and unbound acridine orange (free in solution).**

[0038] In this example, acridine orange was employed as an intercalating molecule to illustrate the differences between the unbound intercalating molecule (free in solution) and double-stranded DNA which is intercalated with the molecule.

[0039] 0.1 mg per ml of calf thymus double-stranded DNA was combined with traces of acridine orange (in sodium acetate buffer, pH 7.0). The acridine orange was obtained from Fisher Chemicals, Fairlawn, New Jersey. After the addition of the acridine, an intensive yellow fluorescence appeared in the reaction mixture.

[0040] As a control, a tube was set up without the aforementioned DNA using traces of acridine orange in sodium acetate buffer, pH 7.0. To the buffered solution was added 5 ul of 0.1 molar of the diazonium salt, p-nitrobenzenediazonium chloride, which was prepared by reducing 4-nitroaniline (Aldrich Chemicals, Milwaukee, WI) to an amine and subsequent diazotization with nitrous acid in aqueous 50% DMSO.

[0041] Reaction was allowed to proceed for five minutes and fluorescence was observed in the reaction solution. After the addition of the diazonium salt, the fluorescence control disappeared, in contrast to the DNA containing solution where the fluorescence persisted. These observations indicate that as an intercalating molecule, acridine orange is protected by the double-stranded DNA, and hints to the wide application of diazonium salts with fluorescent intercalators.

**Claims**

1. A method for detecting the presence of a target polynucleotide in a sample comprising the steps of:

(a) contacting said sample under hybridizing conditions with (i) a single-stranded polynucleotide probe capable of hybridizing to said target polynucleotide, said probe comprising a polynucleotide and at least one intercalating molecule attached to a nucleotide of said polynucleotide by means of a linker arm, wherein upon hybridization of said probe to said target said intercalating molecule induces a detectable change in a property in either said probe, said target polynucleotide or both, and (ii) a background-reducing reagent which chemically modifies said intercalating molecule when the probe attached thereto is single-stranded in such a way that any interfering signal from the unhybridized single stranded DNA probe is minimized or substantially eliminated; and

(b) detecting said property change, thereby detecting said target polynucleotide.

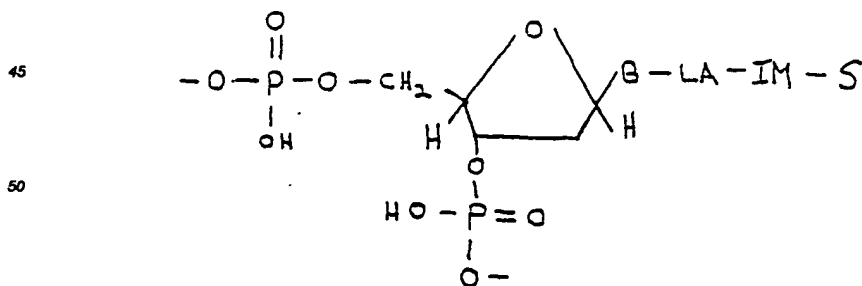
2. The method of claim 1 wherein said target polynucleotide is in double-stranded form and said method further comprises the step of rendering said target polynucleotide in substantially single-stranded form prior to said contacting step.

3. The method of claim 1 or 2 further comprising the step of adding an inactivator which renders said background-reducing reagent inactive prior to detecting the signal.

4. The method of claim 3 wherein said inactivator is selected from the group consisting of an aliphatic primary amine, a phenol and an aromatic amine, or a combination of any of the foregoing.

EP 0 492 570 B1

5. The method according to any one of claims 1 to 4 wherein said property change is selected from the group consisting of radiation emission, interaction of molecular dispersion forces, buoyant density, melting temperature and helical length, or a combination of any of the foregoing.
- 5 6. The method according to any one of claims 1 to 4 wherein said property change comprises a detectable signal.
7. The method according to any one of claims 1 to 6 wherein said intercalating molecule comprises an aromatic dye.
8. The method according to any one of claims 1 to 7 wherein said linker arm is attached to a base moiety of said  
10 polynucleotide probe.
9. The method of claim 8 wherein said base is selected from the group consisting of purines, pyrimidines and deazapurine, or a combination of any of the foregoing.
10. The method of claim 8 or 9 wherein said linker arm comprises at least 3 carbon atoms.
- 15 11. The method according to any one of claims 8 to 10 wherein said linker arm is attached to the base moiety in one fragment.
12. The method according to any one of claims 8 to 11 wherein said linker arm is synthesized by first attaching a first fragment to the base moiety and then attaching a second fragment to the first fragment.
- 20 13. The method according to any one of claims 1 to 12 wherein said background-reducing reagent comprises a diazonium salt.
14. A method for detecting the presence of a target polynucleotide in a sample comprising the steps of:  
25 a) contacting said target polynucleotide with (i) a single-stranded polynucleotide probe, said polynucleotide probe comprising a polynucleotide and at least a first intercalating molecular entity and a second intercalating molecular entity, wherein said first intercalating molecular entity is attached to a first nucleotide of said polynucleotide by means of a first linker arm, and said second intercalating molecular entity is attached to a second nucleotide by means of a second linker arm, said first and said second nucleotides being separated by a number of nucleotides sufficient for the provision of target  
30 specificity to said polynucleotide, said intercalating molecular entities inducing upon hybridization of said polynucleotide probe to said target polynucleotide, a detectable change in a property either in the polynucleotide probe, in the target polynucleotide, or in both; and (ii) a background-reducing reagent which chemically modifies said intercalating molecular entities when the probe attached thereto is single-stranded in such a way that any interfering signal from the unhybridized single stranded DNA probe is minimized or substantially eliminated;  
35 b) forming a hybrid comprising said polynucleotide probe and said target polynucleotide; and  
c) detecting the presence of said target polynucleotide by means of said property change.
- 40 15. A composition comprising a single-stranded polynucleotide probe having at least one moiety with the structure



55 wherein B represents a base selected from the group consisting of pyrimidines, purines and deazapurines, provided that whenever B comprises a pyrimidine, the sugar in said base is attached to the N<sup>1</sup>α-position of the pyrimidine, and whenever B is a purine or a deazapurine, the sugar is attached to the N<sup>9</sup>α-position of the purine or

EP 0 492 570 B1

deazapurine, wherein IM represents an intercalating molecule which upon hybridization of the probe to its target induces a detectable change in a property in either said probe, said target or both, LA represents a linker arm comprising at least three carbon atoms and is attached covalently or non-covalently to said intercalating molecule, and S represents a background-reducing reagent which modifies said intercalating molecule when the probe attached thereto is single-stranded in such a way that any interfering signal from the unhybridized single stranded DNA probe is minimized or substantially eliminated.

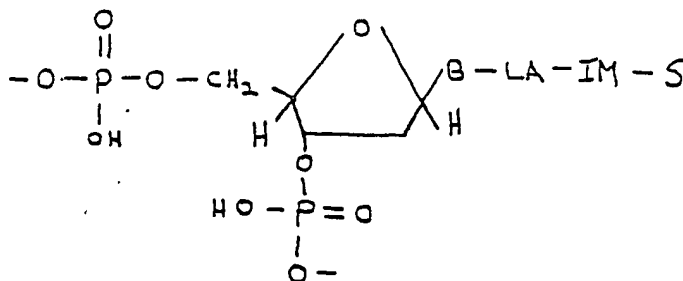
16. The composition of claim 15 wherein said intercalating molecule comprises an aromatic dye.
17. The composition of claim 15 or 16 wherein said background-reducing reagent comprises a diazonium salt.
18. A nucleic acid hybridization kit for detecting the presence of a target polynucleotide in a sample, said kit comprising in one or more containers:
  - (1) a single-stranded polynucleotide probe which is hybridizable to said target polynucleotide, said probe comprising a polynucleotide and at least one intercalating molecule attached to a nucleotide of said polynucleotide by means of a linker arm, wherein said intercalating molecule induces a change in a property in either said probe, said target polynucleotide or both;
  - (2) a background-reducing reagent which modifies said intercalating molecule when the probe attached thereto is single-stranded in such a way that any interfering signal from the unhybridized single stranded DNA probe is minimized or substantially eliminated; optionally with
  - (3) an inactivator which renders the background-reducing reagent inactive.

Patentsprüche

1. Verfahren zum Nachweis des Vorliegens eines Ziel-Polynucleotids in einer Probe, umfassend die Schritte:
  - (a) Inkontaktbringen der Probe unter Hybridisierungsbedingungen mit (i) einer einzelsträngigen Polynucleotidsonde, die zur Hybridisierung mit dem Ziel-Polynucleotid geeignet ist, wobei die Sonde ein Polynucleotid und mindestens ein interkalierendes Molekül umfaßt, das mittels eines Linkerarms an ein Nucleotid des Polynucleotids gebunden ist, wobei nach Hybridisierung der Sonde an das Ziel das interkalierende Molekül eine nachweisbare Veränderung der Eigenschaften in der Sonde, dem Ziel-Polynucleotid oder in beiden hervorruft, und (ii) ein den Hintergrund reduzierendes Reagens, das das interkalierende Molekül chemisch modifiziert, wenn die daran gebundene Sonde einzelsträngig ist, indem jedes interferierende Signal von der nicht-hybridisierten einzelsträngigen DNA-Sonde minimiert oder im wesentlichen eliminiert wird; und
  - (b) Nachweis der Veränderung der Eigenschaft und dadurch Nachweis des Ziel-Polynucleotids.
2. Verfahren nach Anspruch 1, wobei das Ziel-Polynucleotid in doppelsträngiger Form vorliegt und das Verfahren ferner den Schritt der Umwandlung des Ziel-Polynucleotids in eine im wesentlichen einzelsträngige Form, vor dem Schritt des Inkontaktbringens, umfaßt.
3. Verfahren nach Anspruch 1 oder 2 ferner umfassend den Schritt der Hinzugabe eines Inaktivators, der zur Inaktivierung des den Hintergrund reduzierenden Reagens führt, vor Nachweis des Signals.
4. Verfahren nach Anspruch 3, wobei der Inaktivator gewählt ist aus der Gruppe, die aus einem primärem aliphatischem Amin, einem Phenol und einem aromatischem Amin oder einer Kombination jeder der vorher genannten besteht.
5. Verfahren nach einem der Ansprüche 1 bis 4, wobei die Veränderung der Eigenschaft gewählt ist aus der Gruppe, die aus Strahlungsemission, Interaktion von molekularen Dispersionskräften, Schwebedichte, Schmelztemperatur und helikaler Länge, oder einer Kombination jeder der vorher genannten besteht.
6. Verfahren nach einem der Ansprüche 1 bis 4, wobei die Eigenschaftsveränderung ein nachweisbares Signal umfaßt.
7. Verfahren nach einem der Ansprüche 1 bis 6, wobei das interkalierende Molekül einen aromatischen Farbstoff umfaßt.
8. Verfahren nach einem der Ansprüche 1 bis 7, wobei der Linkerarm an eine Baseneinheit der Polynucleotidsonde gebunden ist.

EP 0 492 570 B1

9. Verfahren nach Anspruch 8, wobei die Base gewählt ist aus der Gruppe, die aus Purinen, Pyrimidinen, Deazapurin, oder einer Kombination jeder der vorher genannten besteht.
10. Verfahren nach Anspruch 8 oder 9, wobei der Linkerarm mindestens drei Kohlenstoffatome umfaßt.
11. Verfahren nach einem der Ansprüche 8 bis 10, wobei der Linkerarm an die Baseneinheit in einem Fragment gebunden ist.
12. Verfahren nach einem der Ansprüche 8 bis 11, wobei der Linkerarm synthetisiert wird, indem zuerst ein erstes Fragment an die Baseneinheit gebunden wird und dann ein zweites Fragment an das erste Fragment gebunden wird.
13. Verfahren nach einem der Ansprüche 1 bis 12, wobei das den Hintergrund reduzierende Reagens ein Diazoniumsalz umfaßt.
14. Verfahren zum Nachweis des Vorliegens eines Ziel-Polynucleotids in einer Probe, umfassend die Schritte:
- Inkontaktbringen des Ziel-Polynucleotids mit (i) einer einzelsträngigen Polynucleotidsonde, wobei die Polynucleotidsonde ein Polynucleotid und mindestens eine erste interkalierende molekulare Einheit und eine zweite interkalierende molekulare Einheit umfaßt, wobei die erste interkalierende molekulare Einheit über einen ersten Linkerarm an ein erstes Nucleotid des Polynucleotids gebunden ist und die zweite interkalierende molekulare Einheit über einen zweiten Linkerarm an ein zweites Nucleotid gebunden ist, wobei das erste und zweite Nucleotid durch eine Reihe von Nucleotiden getrennt sind, die ausreichend für die Gewährleistung von Zielspezifität an das Polynucleotid ist, wobei die interkalierenden molekularen Einheiten nach Hybridisierung der Polynucleotidsonde an das Ziel-Polynucleotid eine nachweisbare Veränderung in einer Eigenschaft in der Polynucleotidsonde, in dem Ziel-Polynucleotid oder in beiden induzieren, und (ii) einem den Hintergrund reduzierenden Reagens, das die interkalierenden molekularen Einheiten, wenn die daran bindende Sonde einzelsträngig ist, in einer Art und Weise chemisch modifiziert, daß jedes interferierende Signal von der nicht hybridisierten, einzelsträngigen DNA-Sonde minimiert oder im wesentlichen eliminiert wird;
  - Erzeugen eines Hybrids, umfassend die Polynucleotidsonde und das Ziel-Polynucleotid; und
  - Nachweis des Vorliegens des Ziel-Polynucleotids mit Hilfe der Eigenschaftsveränderung.
15. Zusammensetzung, umfassend eine einzelsträngige Polynucleotidsonde, die mindestens eine Einheit mit der folgenden Struktur besitzt,



- wobei B eine Base ist, ausgewählt aus der Gruppe bestehend aus Pyrimidinen, Purinen und Deazapurin repräsentiert, mit der Maßgabe, daß wenn immer B ein Pyrimidin umfaßt, der Zucker in der Base an die N<sup>1</sup>-Position des Pyrimidins gebunden ist, und wenn immer B ein Purin oder Deazapurin ist, der Zucker an die N<sup>9</sup>-Position des Purins oder Deazapurins gebunden ist, wobei IM ein interkalierendes Molekül repräsentiert, das nach Hybridisierung der Sonde an ihr Ziel eine nachweisbare Veränderung der Eigenschaften in entweder der Sonde, dem Ziel oder in beiden induziert, LA einen Linkerarm repräsentiert, der mindestens drei Kohlenstoffatome umfaßt und kovalent oder nicht-kovalent an das interkalierende Molekül gebunden ist, und S ein den Hintergrund reduzierendes Reagens repräsentiert, das das interkalierende Molekül, wenn die daran bindende Sonde einzelsträngig ist, in einer Art und Weise modifiziert, daß jedes interferierende Signal von der nicht-hybridisierten, einzelsträngigen DNA-Sonde minimiert oder im wesentlichen eliminiert wird.
16. Zusammensetzung nach Anspruch 15, wobei das interkalierende Molekül einen aromatischen Farbstoff umfaßt.

EP 0 492 570 B1

17. Zusammensetzung nach Anspruch 15 oder 16, wobei das den Hintergrund reduzierende Reagens ein Diazoniumsalz umfaßt.
- 5 18. Nucleinsäure-Hybridisierungs-Kit zum Nachweis des Vorliegens eines Ziel-Polynucleotids in einer Probe, wobei der Kit ein oder mehrere Behältnisse umfaßt:
- 10 (1) eine einzelsträngige Polynucleotidsonde, die hybridisierbar an das Ziel-Polynucleotid ist, wobei die Sonde ein Polynucleotid und mindestens ein interkalierendes Molekül umfaßt, das mittels eines Linkerarms an ein Nucleotid des Polynucleotids gebunden ist, wobei das interkalierende Molekül eine Eigenschaftsveränderung in entweder der Sonde, dem Ziel-Polynucleotid oder in beiden induziert;
- (2) ein den Hintergrund reduzierendes Reagens, das das interkalierende Molekül, wenn die daran gebundene Sonde einzelsträngig ist, in einer Art und Weise modifiziert, daß jedes interferierende Signal von der nicht-hybridisierten einzelsträngigen DNA-Sonde minimiert oder im wesentlichen eliminiert wird; gegebenenfalls mit
- 15 (3) einem Inaktivator, der zur Inaktivierung des den Hintergrund reduzierenden Reagens führt.

Revendications

- 20 1. Procédé pour détecter la présence d'un polynucléotide cible dans un échantillon, qui comprend les étapes
- (a) de mise en contact dudit échantillon dans les conditions d'une hybridation (i) avec une sonde polynucléotidique simple brin capable de s'hybrider audit polynucléotide cible, ladite sonde comprenant un polynucléotide et au moins une molécule d'intercalation fixée à un nucléotide dudit polynucléotide au moyen d'un bras de liaison, ladite molécule d'intercalation provoquant, après hybridation de ladite sonde à ladite cible, un changement détectable d'une propriété de ladite sonde, dudit polynucléotide cible, ou des deux, et (ii) avec un réactif réducteur de fond, qui modifie chimiquement ladite molécule d'intercalation quand la sonde qui y est fixée est rendue simple brin de telle sorte que tout signal interférant provenant de la sonde à ADN simple brin non-hybridée soit minimisé ou pour ainsi dire éliminé ; et
- 25 (b) de détection dudit changement de propriété, de façon à détecter ledit polynucléotide cible.
- 30 2. Procédé selon la revendication 1, dans lequel ledit polynucléotide cible se présente sous forme double brin, et ledit procédé comprend en outre l'étape consistant à donner audit polynucléotide cible une forme essentiellement simple brin avant ladite étape de mise en contact.
- 35 3. Procédé selon la revendication 1 ou 2, qui comprend en outre l'étape d'addition d'un inactivateur, qui rend inactif ledit agent réducteur de fond avant la détection du signal.
- 40 4. Procédé selon la revendication 3, dans lequel ledit inactivateur est choisi dans l'ensemble comprenant une amine primaire aliphatique, un phénol et une amine aromatique, ou une combinaison d'un ou plusieurs quelconques de ces derniers.
- 45 5. Procédé selon l'une quelconque des revendications 1 à 4, dans lequel ledit changement de propriété est choisi dans l'ensemble comprenant l'émission d'un rayonnement, l'interaction des forces de dispersion moléculaire, l'équilibre de sédimentation dans un gradient de densité, la température de fusion et la longueur de l'hélice, ou une combinaison d'un ou plusieurs quelconque de ces derniers.
- 50 6. Procédé selon l'une quelconque des revendications 1 à 4, dans lequel ledit changement de propriété comprend un signal détectable.
7. Procédé selon l'une quelconque des revendications 1 à 6, dans lequel ladite molécule d'intercalation comprend un colorant aromatique.
- 55 8. Procédé selon l'une quelconque des revendications 1 à 7, dans lequel ledit brin de liaison est fixé à une portion de base de ladite sonde polynucléotidique.
9. Procédé selon la revendication 8, dans lequel ladite base est choisie dans l'ensemble comprenant les purines, les pyrimidines et les désazapurines, ou une combinaison d'une ou plusieurs quelconque de ces dernières.
10. Procédé selon la revendication 8 ou 9, dans lequel ledit bras de liaison comprend au moins 3 atomes de carbone.
11. Procédé selon l'une quelconque des revendications 8 à 10, dans lequel ledit bras de liaison est fixé à la

portion de base d'un fragment.

12. Procédé selon l'une quelconque des revendications 8 à 11, dans lequel ledit bras de liaison est synthétisé d'abord par fixation d'un premier fragment à la portion de base, puis par fixation d'un deuxième fragment au premier fragment.

13. Procédé selon l'une quelconque des revendications 1 à 12, dans lequel ledit réactif réducteur de fond comprend un sel de diazonium.

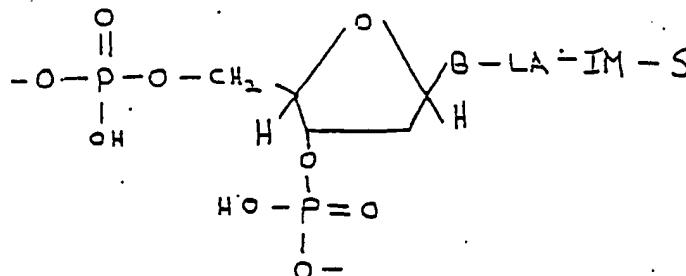
14. Procédé pour détecter la présence d'un polynucléotide cible dans un échantillon, qui comprend les étapes

a) de mise en contact dudit polynucléotide cible (i) avec une sonde polynucléotidique simple brin, ladite sonde polynucléotidique comprenant un polynucléotide et au moins une première entité moléculaire d'intercalation et une deuxième entité moléculaire d'intercalation, ladite première entité moléculaire d'intercalation étant fixée à un premier polynucléotide dudit polynucléotide au moyen d'un premier bras de liaison, et ladite deuxième entité moléculaire d'intercalation étant fixée à un deuxième nucléotide au moyen d'un deuxième bras de liaison, ledit premier et ledit deuxième nucléotides étant séparés par un nombre de nucléotides suffisant pour conférer audit polynucléotide une spécificité de cible, lesdites entités moléculaires d'intercalation provoquant, après hybridation de ladite sonde polynucléotidique audit polynucléotide cible, un changement détectable d'une propriété dans la sonde polynucléotidique, dans le polynucléotide cible, ou dans les deux, et (ii) avec un réactif réducteur de fond, qui modifie chimiquement lesdites entités moléculaires d'intercalation quand la sonde qui y est fixée est rendue simple brin, de telle sorte que tout signal interférant provenant de la sonde à ADN simple brin non-hybridée soit minimisé ou pour ainsi dire éliminé ;

b) de formation d'un hybride comprenant ladite sonde polynucléotidique et ledit polynucléotide cible ; et

c) de détection de la présence dudit polynucléotide cible au moyen dudit changement de propriété.

15. Composition comprenant une sonde polynucléotidique simple brin ayant au moins une portion présentant la structure :



dans laquelle B représente une base choisie dans l'ensemble comprenant les pyrimidines, les purines et les désazapurines; du moment que, quand B comprend une pyrimidine, le sucre de ladite base est fixé à la position N<sup>1</sup> de la pyrimidine, et, quand B est une purine ou une désazapurine, le sucre est fixé à la position N<sup>9</sup> de la purine ou de la désazapurine, où IM représente une molécule d'intercalation qui, après hybridation de la sonde à sa cible, provoque un changement détectable d'une propriété dans ladite sonde, dans ladite cible ou dans les deux, LA représente un bras de liaison comprenant au moins trois atomes de carbone et est fixé d'une manière covalente ou non-covalente à ladite molécule d'intercalation, et S représente un réactif réducteur de fond; qui modifie ladite molécule d'intercalation quand la sonde qui y est fixée est rendue simple brin, de telle sorte que tout signal interférant provenant de la sonde à ADN simple brin non-hybridée soit minimisé ou pour ainsi dire éliminé.

16. Composition selon la revendication 15, dans lequel ladite molécule d'intercalation comprend un colorant aromatique.

17. Composition selon la revendication 15 ou 16, dans lequel ledit réactif réducteur de fond comprend un sel de diazonium.

18. Trousse d'hybridation d'acides nucléiques pour détecter la présence d'un polynucléotide cible dans un échantillon, ladite trousse comprenant, un ou plusieurs réceptifs :



**EP 0 492 570 B1**

(1) une sonde polynucléotidique simple brin qui est hybridable audit polynucléotide cible, ladite sonde comprenant un polynucléotide et au moins une molécule d'intercalation fixée à un nucléotide dudit polynucléotide au moyen d'un bras de liaison, où ladite molécule d'intercalation provoque un changement d'une propriété dans ladite sonde, dans ledit polynucléotide cible, ou dans les deux ;

5

(2) un réactif réducteur de fond, qui modifie ladite molécule d'intercalation quand la sonde qui y est fixée est rendue simple brin de telle manière que tout signal interférant provenant de la sonde à ADN simple brin non-hybridée soit minimisé ou pour ainsi dire éliminé ; éventuellement avec

10

(3) un inactivateur, qui rend inactif le réactif réducteur de fond.

15

20

25

30

35

40

45

50

55